VARYING INFLUENCE OF TUBERCULOUS RABBIT PLASMA ON THE GROWTH OF FIBROBLASTS IN VITRO

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In studying tuberculin reactions by means of tissue cultures attempts were made to "sensitize" normal fibroblasts *in vitro*. It soon appeared that plasma from rabbits with early tuberculosis had a different primary influence from that obtained later in the disease; hence these differences were more carefully investigated. This report deals with the results of that investigation.

EXPERIMENTAL

Animals.—In an attempt to eliminate the variables due to differences of age and sex, litters of known age were obtained from a stock of rabbits bred in the Institute. The animals were so selected that a tuberculous and a non-tuberculous litter mate of the same sex were kept as pairs; the tuberculous animals were housed in a different room from the controls, but were given a similar diet. The type of rabbit used—hybrid of English, lilac and Havana—has been subsequently shown to be relatively highly resistant to tuberculosis; hence the disease probably ran a more chronic course than usual. At the time of inoculation the animals were immature and varied from 1,500 gm. to 1,800 gm. in weight. The majority of them lived from 5 to 10 months after inoculation, some over a year; they succumbed finally to the chronic form of the disease, often after months of testicular, arthritic or ocular tuberculosis.

Inoculum.—The rabbits were inoculated intravenously with 0.1 mg. of an 11 to 13 day culture of a bovine strain of tubercle bacilli, B 1, grown on Petroff's gentian violet egg medium. It has subsequently been shown that these cultures probably contained a mixture of smooth and rough forms. The growth was harvested from the surface, weighed wet, then ground in a mortar with Ringer's solution until a uniform suspension resulted. The coarser granules were removed by slow centrifugalization for 3 minutes. The subsequent suspension was suitably diluted so that 0.5 cc. contained 0.1 mg. of bacilli. This amount was injected into the marginal ear vein; and in all but one animal these veins remained free of tuberculous lesions.

Tissue Cultures.—The cells used for testing the influence of the plasma were so called fibroblasts grown from the testes of adolescent rabbits. Under aseptic precautions, the testes were removed from animals immediately post mortem or while under full ether anesthesia, cut into small bits, of which four pieces were explanted into a medium consisting of heparinized rabbit plasma and tissue extract. They were washed and renourished twice a week with plasma or tissue extract; after 10 to 14 days healthy appearing growths were divided into four pieces and transplanted into similar medium. At the time of an actual experiment each transplant was carefully excised from the medium in which it was growing, divided into four equal sized portions, each of which was placed in a different Carrel culture flask. Four such transplants from four different original pieces were thus placed in similarly marked segments of four different flasks. With this arrangement the subcultures from two of these stock transplants were used as controls and two as the objects for testing the influence of the abnormal environment. Under these conditions the factor of varying initial growth energy was controlled as well as possible, for by identifying each subculture with a distinctive mark each of the four could be compared with its fellows having a common source.

Plasma.—Except in one or two instances where serum was employed the fluid containing the growth-influencing substances consisted of plasma prepared as follows: Blood was allowed to drop from a small incision in the marginal ear vein of the rabbit directly into tubes containing 0.5 cc. of 0.1 per cent heparin dissolved in Ringer's solution until a total volume of 4 cc. was obtained. The tubes were placed immediately in ice, and centrifuged after all of the specimens had been collected. The supernatant plasma was pipetted into other tubes and kept cold until used in the cultures.

Tissue Extract.—Three different extracts were used, two in preliminary studies and the third in the main portion of the work. At first, chick embryonic extract was tried, but soon proved to be inferior in stimulation of rabbit fibroblasts as compared with homologous extracts; moreover, when heterologous extracts were used the effect of growth inhibiting substances was accentuated. Rabbit splenic extract was more satisfactory, but was much more costly than rabbit embryonic extract, since larger amounts of the latter could be obtained from one animal. 16 day rabbit embryos were separated from the placentae and membranes, weighed and passed through a Fischer embryonic tissue crusher, then mixed with enough Tyrode's solution to make a 25 per cent suspension. This was centrifuged and the supernatant turbid fluid used as the plasma-coagulating reagent. Unless this extract were to be employed within a few days it was preserved in a frozen state, for in this condition it appeared to retain its activity for weeks.

In most of the actual experiments the cultures were set up in two phases: solid and liquid. After the four selected transplants were placed in the four quadrants respectively of a Carrel D or micro flask containing 0.5 cc. of heparinized normal rabbit plasma, 0.25 cc. of 25 per cent tissue or embryonic extract was added; then the flasks were transferred to a warm stage at 37°C. in order to facilitate coagulation of the plasma. Thus far, the environmental conditions of the transplants

in a given experiment were common to all. The flasks were now arranged in pairs so that each pair contained transplants from a common source. Excess of the embryonic extract was removed by placing 0.5 cc. of Tyrode's solution in each flask, allowing to stand half an hour, then pipetting it off; this maneuver was repeated. Then to one of each pair of flasks was added 0.25 cc. of plasma from a tuberculous rabbit and 0.25 cc. of Tyrode's solution, and to the other similarly diluted plasma from a normal sibling of the same sex. The flasks were incubated at 37°C. and examined daily. On the 3rd or 4th day they were opened, washed with Tyrode's solution, as described above, and 0.5 cc. of fresh 50 per cent plasma from the same rabbits as previously used, was added to each. Again, they were tightly stoppered and returned to the incubator.

In the last two experiments the procedure was modified somewhat: Instead of employing a solid phase of normal plasma and a liquid phase of tuberculous or control plasma, the transplants were placed immediately in 0.7 cc. of the tuberculous or non-tuberculous plasma respectively, and 0.3 cc. of 25 per cent rabbit embryonic extract. As soon as the coagulum was solid the flasks were stoppered and placed in the incubator without being subjected to the maneuver of washing and adding fresh plasma. Subsequently, however, these cultures were washed and renourished with 50 per cent plasma as described above.

Estimation of Growth.—The cultures were examined microscopically at frequent intervals in order to compare the appearance of the cells growing under different environments. The amount of increase in growth was recorded according to the method of Ebeling (1) by means of outline drawings made with the aid of an Edinger projectoscope. The areas thus outlined were measured with a planimeter. In each experiment from 8 to 16 transplants were subjected to the influence of the abnormal plasma, with a corresponding number of controls. The total areas of all the transplants growing under any particular environment on a given day were combined and from these totals the increments of growth were estimated. In this way the errors resulting from individual variations in rates of growth and inherent in the method were reduced to a minimum. Infected cultures and those in which the growth was obviously influenced by factors other than those experimentally introduced were eliminated from the calculations. The influence of the abnormal plasma is expressed by the ratio $\frac{\text{Tuberculous plasma}}{\text{Normal plasma}}$; when distinctly less than 1 it indicates that growth-depressing influences in the environment predominated, and, when more than 1, that growth-stimulating factors were dominant.

Experimental Results

The results obtained in eighteen different experiments are combined and set out in detail in Table I. The increments of growth on the days indicated are shown for each experiment; the data obtained with tuberculous plasma are given first for each pair, and those ob-

TABLE I

Comparative Rates of Growth of Fibroblasts in Tuberculous and Normal Plasma

Experiment No.	Rabbit No.	Condition	Duration of tuberculosis		Day 2	3	4	5	6	7	8	9	10	11
T 37	3 pooled*	Tb	21 days	Increment	3.7	11.0	25.4	31.0						
	3 " *	Normal		"	3.5	10.5	31.0	48.5						
				Ratio $\frac{Tb}{Nl}$	*1.06*	*1.05*	*0.84*	*0.64*						
T 63	34-65	Tb	18 days	Increment	1.5	4.7	10.6	17.4	25.4		35.0			
	34-71	Normal		"	1.5	5.1	11.7	16.4	21.0		36.2			
				Ratio $\frac{Tb}{Nl}$	*1.0*	*0.92*	*0.91*	*1.06*	*1.2*		*0.97*			
T 65 *a*	34-67	Tb	24 days	Increment	0.52	1.5	2.6	5.1			11.0	14.6		25.0
	34-71	Normal		"	0.51	1.3	2.7	6.8			20.0	27.3		48.0
				Ratio $\frac{Tb}{Nl}$	*1.0*	*1.15*	*0.97*	*0.75*			*0.55*	*0.53*		*0.52*
T 65 *b*	34-65	Tb	24 days	Increment	0.53	1.7	3.1	7.5			16.6	23.1		35.5
	34-69	Normal		"	0.44	1.9	3.4	8.0			21.5	28.4		41.6
				Ratio $\frac{Tb}{Nl}$	*1.2*	*0.9*	*0.91*	*0.94*			*0.77*	*0.81*		*0.85*
T 66	34-65	Tb	31 days	Increment	1.2	3.6		8.6	16.7		32.2			
	34-69	Normal		"	2.0	10.6		18.5	32.6		50.0			
				Ratio $\frac{Tb}{Nl}$	*0.6*	*0.3*		*0.46*	*0.51*		*0.64*			

* Serum was used instead of plasma.

T 67	34-67	Tb	31 days	Increment	0.58		2.3	5.0		11.3				
	34-71	Normal		"	1.5		5.5	10.3		18.8				
				Ratio $\frac{Tb}{Nl}$	*0.39*		*0.42*	*0.48*		*0.60*				
T 68	34-65	Tb	38 days	Increment	0.18	1.4	4.9		12.5		20.0			31.0
	34-69	Normal		"	0.11	0.9	4.7		10.0		15.6			27.0
				Ratio $\frac{Tb}{Nl}$	*1.6*	*1.56*	*1.04*		*1.25*		*1.28*			*1.15*
T 71	34-67	Tb	66 days	Increment	2.1	4.2	6.5			21.2		41.0		
	34-71	Normal		"	2.1	7.4	8.5			24.4		45.0		
				Ratio $\frac{Tb}{Nl}$	*1.0*	*0.57*	*0.77*			*0.87*		*0.91*		
T 72	34-67	Tb	73 days	Increment	0.4	2.9	5.2		8.7					
	34-71	Normal		"	0.44	2.7	4.3		8.4					
				Ratio $\frac{Tb}{Nl}$	*0.91*	*1.07*	*1.21*		*1.03*					
T 79 *a*	34-70	Tb	6 wks.	Increment	1.1	3.6	6.8		17.4		31.0		40.2	
	34-71	Normal		"	1.3	5.1	9.5		23.8		42.7		52.6	
				Ratio $\frac{Tb}{Nl}$	*0.85*	*0.71*	*0.72*		*0.73*		*0.74*		*0.76*	
T 79 *b*	34-65	Tb	4 mos.	Increment	1.4	4.2	7.7		19.2		32.7		47.0	
	34-71	Normal		"	1.3	5.1	9.5		23.8		42.7		52.6	
				Ratio $\frac{Tb}{Nl}$	*1.08*	*0.82*	*0.81*		*0.81*		*0.77*		*0.9*	
T 79 *c*	32-86	Tb	9 mos.	Increment	0.67	2.6	5.7		13.5		22.4		34.3	
	32-88	Normal		"	0.54	2.3	4.9		11.7		22.0		32.0	
				Ratio $\frac{Tb}{Nl}$	*1.24*	*1.13*	*1.16*		*1.16*		*1.02*		*1.07*	

TABLE I—*Concluded*

Experiment No.	Rabbit No.	Condition	Duration of tuberculosis		Day 2	3	4	5	6	7	8	9	10	11
T 88 *a*	36-25	Tb	16 days	Increment		2.6		4.6		8.7				
	36-26	Normal		"		3.5		9.4		22.5				
				Ratio $\frac{Tb}{Nl}$		*0.74*		*0.49*		*0.39*				
T 88 *b*	34-67	Tb	6 mos.	Increment		2.0		5.6		13.4				
	34-69	Normal		"		3.7		10.6		28.0				
				Ratio $\frac{Tb}{Nl}$		*0.54*		*0.53*		*0.48*				
T 88 *c*	32-86	Tb	11 mos.	Increment		3.5		8.0		17.0				
	32-88	Normal		"		2.3		5.8		17.0				
				Ratio $\frac{Tb}{Nl}$		*1.52*		*1.38*		*1.0*				
T 89 *a*	36-25	Tb	3 wks.	Increment	1.8	4.7	6.5		9.0		11.4			
	36-26	Normal		"	1.9	4.6	7.2		11.4		17.4			
				Ratio $\frac{Tb}{Nl}$	*0.95*	*1.0*	*0.91*		*0.79*		*0.66*			
T 89 *b*	34-67	Tb	6 mos.	Increment	2.2	7.4	11.0		19.2		28.2			
	34-69	Normal		"	1.9	6.0	9.1		13.8		19.6			
				Ratio $\frac{Tb}{Nl}$	*1.16*	*1.24*	*1.2*		*1.39*		*1.42*			
T 89 *c*	32-86	Tb	11 mos.	Increment	1.8	5.3	8.7		25.0		58.0			
	32-88	Normal		"	1.9	6.6	10.0		15.0		26.0			
				Ratio $\frac{Tb}{Nl}$	*1.05*	*0.8*	*0.87*		*1.67*		*2.23*			

tained with the plasma of its normal sibling are given second. The ratio $\frac{\text{Tuberculous plasma}}{\text{Normal plasma}}$ is indicated in italics. Excepting in the first experiment (T 37) all the growths were observed for a week or longer; for it soon appeared that the differences in the influence of the two types of plasma were more evident after a week than during the first few days following transplantation.

The rates of the increment of growth varied widely in the different experiments. This is not surprising in view of the fact that different stocks of transplants were employed at various times. During a certain period the stock was nourished simply with normal plasma, and under these conditions it became evident that the growth energy gradually decreased. With cultures of distinctly poor initial growth energy the depressing influence of tuberculous plasma was so marked that significant growth curves could not be obtained; such experiments were, therefore, eliminated from the final reckoning. When, on the other hand, the stock transplants were nourished with embryonic extract their growth was more vigorous and after being transplanted and subjected to the experimental environments the differences were brought out with significant clarity.

In Experiments T 63 and T 88 *a* the plasma was obtained on the 18th and 16th days of tuberculosis respectively. It was very turbid and, after cooling and centrifuging, showed a creamy layer. After a few days' growth the fibroblasts nourished in this medium were so filled with yellowish brown granules that they gave the impression of dying cells. They continued to grow, however, and concomitantly the plasma immediately surrounding the transplants became clearer. One seemed justified in concluding, therefore, that the growing cells were phagocytosing lipoidal substances from the tuberculous plasma. With the passage of weeks the plasma from the tuberculous rabbits became progressively clearer, until by the 6th or 7th week it had practically the same macroscopic appearance as normal plasma. During this time, however, the cells growing in the plasma from the diseased animals continued to show a more granular appearance, so that they could easily be distinguished from those growing under a normal environment. Cells grown in tuberculous plasma obtained after the 4th month, on the other hand, had almost the same microscopic

appearance as those grown in normal plasma. The possible significance of these observations is considered in the discussion.

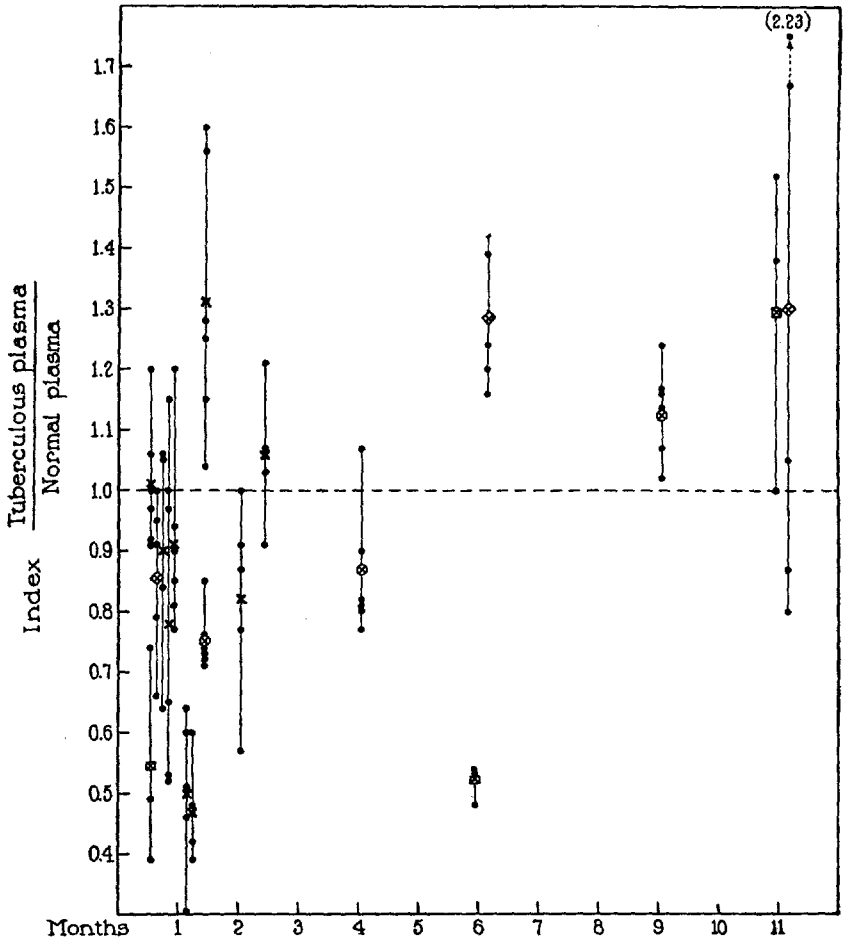

CHART 1. Comparative indices of growth in plasma from rabbits in various stages of tuberculosis.

×'s represent average, and the dots individual observations; the lines indicate the variations in a given experiment.

× in circle indicates Experiment T 79, *a*, *b*, *c*, respectively.

× in square indicates Experiment T 88, *a*, *b*, *c*, respectively.

× in diamond indicates Experiment T 89, *a*, *b*, *c*, respectively.

The averages of the ratios $\frac{\text{Tuberculous plasma}}{\text{Normal plasma}}$ are most easily compared in graphic form as in Chart 1. These averages, indicated by $\times$, were estimated from all the ratios in each individual experiment; the range for each experiment is indicated by a vertical line, and each ratio is indicated by a dot on the line. Similar symbols are used to indicate experiments where three different plasmas were tested simultaneously with the same stock of transplants. While this form may not be absolutely correct from a statistical viewpoint, it represents fairly well the varying influences of tuberculous plasma obtained at different periods in the evolution of the disease in rabbits.

This chart demonstrates that most of the average indices obtained with plasma from tuberculous rabbits in the first 3 months of the disease were below 1, while most of those from rabbits having had tuberculosis 6 to 11 months were above 1. Some unexplained exceptions to the rule were observed. In two experiments, Nos. T 68 and T 72, the plasma was 4 days old before it was used for the nourishment of the fibroblasts; in the former the index was always above 1 and was the highest that was seen with plasma in the early period; in the latter the index hovered close to 1. It is possible that during the 4 day period in the ice box the plasma had lost some of its inhibitory properties. In Experiment T 63, where the index was practically 1, the areas of total growth may have given a false impression of the relative number of cells in the transplants. Microscopic examination showed that most of the cells growing in this very turbid tuberculous plasma were so stuffed with lipoidal granules that the individual cells were nearly twice as large as those growing in the normal control plasma. Had they been the same size probably the areas occupied by the total growths would have been considerably smaller with a corresponding decrease in the index.

In Experiment T 88 *b* the stock of fibroblasts was far from vigorous, so that even a small amount of inhibitory substance in the plasma was apparently reflected in the growth curves. This was shown also in Experiments T 88 *a* and *c* that were carried on at the same time. In all instances where the cells were exposed to tuberculous plasma there were distinct microscopic evidences of toxic action of the tuberculous environment. Nevertheless, the differences between the plasmas ob-

tained very early and relatively late in tuberculosis are still brought out in these two sets. In Experiment T 89 the plasma obtained 1 week later from the same rabbits and tested on a new stock of vigorously growing fibroblasts gave results more in keeping with the general rule; here the index with plasma from rabbits suffering from tuberculosis for 6 months was well above 1.

The question naturally arose as to whether the poorer growth in an environment of tuberculous plasma was the result of an increase in inhibitory substances or a diminution in growth-stimulating factors. The fact that, as a rule, the early periods of the tuberculosis occurred

TABLE II

Comparative Rates of Growth of Fibroblasts in Plasma and Serum of Tuberculous Rabbits

Experiment No.	Medium		Day 2	3	4	5	6	7	8
T 66 *x*	Tb plasma	Increment	1.2	3.6		8.6	16.7		32.0
	Tb serum	"	1.1	4.8		18.0	38.0		66.0
		Ratio $\frac{Plasma}{Serum}$	*1.1*	*0.75*		*0.48*	*0.44*		*0.49*
T 67 *x*	Tb plasma	Increment	0.58		2.3	5.0		11.0	
	Tb serum	"	1.5		8.3	16.7		40.0	
		Ratio $\frac{Plasma}{Serum}$	*0.39*		*0.28*	*0.3*		*0.28*	

during the youth of the animals while the late periods represented maturity suggested that inhibitory substances were responsible for depression of growth; because it is well known that growth-inhibitory substances increase *pari passu* with age (2). Additional evidence pointing in the same direction was furnished by comparing the action of plasma and serum obtained at the same period from tuberculous animals. The increments in growth and the indices of $\frac{\text{Plasma}}{\text{Serum}}$ are shown in Table II. It is obvious that there were more growth-inhibitory factors in tuberculous plasma than in tuberculous serum. Parallel experiments with normal plasma and serum also showed that the former was much more inhibitory than the latter. Unfortunately,

some of the normal serum controls became contaminated, so that the indices could not be completely estimated. Experiment T 37 (Table I) indicates, however, that serum from rabbits early in tuberculosis is more inhibitory than is that from normal controls. Microscopically the cells grown in tuberculous serum or plasma (Experiments T 66 *x* and T 67 *x*) became equally granular, much more so than cells grown in normal serum or plasma. Whether this granulation was the result of a toxic action of the plasma on the cytoplasm or of phagocytosed lipoid particles is impossible to state.

DISCUSSION

Two distinct effects from growing fibroblasts in plasma from tuberculous rabbits were obvious: the first, occurring in the initial stages of the disease, was growth-inhibitory; the second, developing during late tuberculosis, was growth-stimulating. These two effects are roughly parallel with the diphasic nature of tuberculosis observed by Thomas (3) in rabbits inoculated intravenously with the same strain of tubercle bacilli used by us.[1] Because we employed a constant and relatively small dose of tubercle bacilli, and a single breed of rabbits, the course of the infection was fairly constant. With one or two exceptions all of the animals passed into the chronic stage of the disease.[2] For this reason it was impossible for us to state absolutely the type of lesions during the first 3 months, but one may safely conclude that there were extensive diffuse lesions in the lungs, liver, spleen, lymph nodes and bone marrow, and probably in the kidneys. Clinical observations showed that practically all of the males had tuberculosis of the testicles, several had lesions of the joints and eyes; and postmortem examinations of animals dying after 6 to 12 months revealed relatively few lesions in the lungs, spleen, lymph nodes or liver. The genitourinary tract, on the other hand, was almost constantly involved.

[1] We are indebted to Dr. R. M. Thomas for several of the cultures used. Much of our work was in progress during some of the period covered by his observations. We feel justified, therefore, in comparing the course of the disease observed in the two sets of experiments.

[2] Attempts to use a litter of another variety, *i.e.* English, resulted in early death of all the animals inoculated.

The question naturally arises as to the correlation between the clinical course of the disease and the factors in the plasma that were observed to exert an influence on the growth of fibroblasts *in vitro*. Serum from cachectic animals is known to contain growth-inhibiting substances; but such elements can hardly be invoked here, because the rabbits continued to gain weight during the first few months following inoculation. True, this gain was not so great as that of their normal litter mates; but, in general, the tuberculous animals appeared to be fairly healthy during this period, and only became cachectic towards the end of their life.

A more probable explanation of the depressing influence of fibroblastic growth observed with plasma in the earlier period of tuberculosis is the presence of an excess of lipoids in these plasmas. This was very marked in the 3rd week, and diminished thereafter. Baker and Carrel (4) have shown that the lipoids of serum are the chief growth-depressing components of that fluid; and that the increased growth-inhibiting factors that accompany senescence are associated with an increase of serum lipoids. In our experiments there was no obvious relation between intensity of macroscopic lipoidemia and degree of inhibition. No attempt was made to determine the nature of these lipoids, so one can merely make conjectures concerning their origin. They may have arisen from the diseased tissues, or from the disintegrating tubercle bacilli, or from a combination of both. Other toxic substances may have carried the inhibiting factor, for it is easy to understand how poisons might be released from both pathological tissues and bacterial cells.

The two phases of the influence of plasma on fibroblastic growth may also be compared with the usual course in the blood picture in tuberculous rabbits (Thomas (3)). In the first 2 months there is a leucopenia involving all elements except the monocytes. This is followed by a leucocytosis characterized by a distinct increase in both monocytes and polymorphonuclears. Carrel (5) has designated the leucocytes as the trephocytes of fibroblasts, because growth-stimulating substances can be demonstrated in them. In our experiments growth-inhibitory factors were present in the plasma during the period of leucopenia, and growth-stimulating substances during the stage of distinct leucocytosis. It is, of course, impossible to state

whether the two phenomena have a common basis or are merely concomitant. It is at least of more than passing interest that the stage of tuberculosis in which there is a distinct tendency for the animal to wall off the tuberculous lesions with a thick fibrous capsule should also be characterized by the presence in its plasma of substances which stimulate the growth of fibroblasts to an unusual degree.

SUMMARY

Plasma obtained from tuberculous rabbits within 3 or 4 months following inoculation with bovine tubercle bacilli exerted a growth-inhibitory influence on transplants of rabbit fibroblasts; while that obtained after the 4th month was growth-stimulating. It is suggested that the growth-inhibitory factor was linked in part with lipoidemia; while the growth-stimulating elements were associated with the period of leucocytosis.

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